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# A novel penicillin acylase from the environmental gene pool with improved synthetic properties

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## Abstract

A new penicillin acylase was isolated by cloning and functional screening of DNA isolated from a sand soil enrichment culture. Sequence analysis of this enzyme, PAS2, revealed homology to a group of prominent penicillin G acylases, including the intensively studied enzyme of *Escherichia coli* ATCC 11105. Accordingly, PAS2 was found to be an Ntn-hydrolase with an N-terminal serine as the catalytic nucleophile, located on its 61.9 kDa  $\beta$ -subunit. The  $\alpha$ -subunit was shown to have a molecular mass of 25.5 kDa. To evaluate the biocatalytic performance of the new enzyme, the complex kinetic parameters  $\alpha$ ,  $\beta_0$ , and  $\gamma$  were determined for the kinetically controlled synthesis of a number of important semi-synthetic penicillins and cephalosporins. While  $\alpha$  is a measure for the relative affinity of the enzyme for the activated acyl donor (AD),  $\beta_0$  and  $\gamma$  quantify the efficiency of acyl-transfer to the  $\beta$ -lactam nucleophile. Compared to the penicillin acylase of *E. coli*, PAS2 showed superior potential for the synthesis of 6-aminopenicillanic acid (6-APA)-derived antibiotics, allowing the accumulation of up to 2.3-fold more target product at significantly higher conversion rates. In the synthesis of amoxicillin, for instance, 1.6-fold more antibiotic was formed using the new enzyme, making PAS2 an interesting candidate for biocatalytic application.

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**Keywords:** Penicillin acylase; Environmental gene bank; Synthesis; Enzyme kinetics;  $\beta$ -Lactam antibiotics

## 1. Introduction

Penicillin acylase (penicillin amidase (PA), EC 3.5.1.11) occurs in many bacteria, yeasts and filamentous fungi. Since more than a decade, this enzyme is commercially employed for the large-scale hydrolysis of penicillin G that is fermentatively produced by *Penicillium chrysogenum* [1–4]. The reaction product, 6-aminopenicillanic acid (6-APA), is a key intermediate in the synthesis of clinically useful semi-synthetic penicillins such as ampicillin or amoxicillin.

While the hydrolytic application of PA is thus well established, the synthetic capacity of the enzyme, although already described some 40 years ago [5], is still less exploited due to thermodynamic constraints. Since the reaction equilibrium is unfavorable, yields in the direct condensation of 6-APA with D-phenylglycine (D-PG) and its derivatives are

very low [6]. The same holds true for the production of semi-synthetic cephalosporins such as cephalexin and cefadroxil with 7-aminodesacetoxycephalosporanic acid (7-ADCA) as the  $\beta$ -lactam group.

Yields can be improved by using activated acyl donor (AD) moieties, mostly the amide or ester derivatives of the acid (Fig. 1). In these kinetically controlled reactions, significantly higher antibiotic concentrations can transiently be reached during the conversion process. However, yields are still limited due to two enzyme-catalyzed side-reactions: (1) the hydrolysis of the activated acyl donor and (2) the hydrolysis of the synthesized antibiotic. Due to the undesired hydrolytic reactions, the unproductive loss of acyl donor will exceed the accumulation of antibiotic in the course of the conversion, which is a major drawback for an industrial process. Many studies have been aimed at improving the kinetically controlled synthesis of semi-synthetic  $\beta$ -lactam antibiotics by medium engineering or modifying the reaction conditions. This includes optimizing the pH [7], addition of cosolvents

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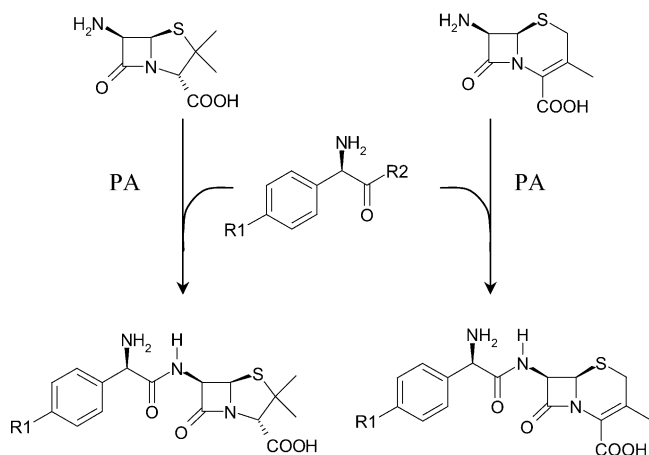


Fig. 1. The use of penicillin acylase in the synthesis of semi-synthetic penicillins (left: ampicillin, R1=H; amoxicillin, R1=OH) and cephalosporins (right: cephalixin, R1=H; cefadroxil, R1=OH). In kinetically controlled synthesis reactions, an activated derivative of the acyl-donor is used, most commonly the amide (R2=NH<sub>2</sub>) or methyl ester (R2=OCH<sub>3</sub>) of phenylglycine (R1=H) and *p*-hydroxyphenylglycine (R1=OH), respectively.

[8], or the use of high substrate concentrations [9] as well as improvement of the biocatalyst (e.g. by immobilization [10]).

A more fundamental approach to enhance product yields is the use of new biocatalysts with improved kinetic properties, since it has been shown that kinetically controlled reactions are fundamentally influenced by the kinetic parameters of the employed enzyme [11,12]. The availability of a penicillin acylase with outstanding synthetic performance is thus a key factor in the development of biocatalytic processes for the synthesis of semi-synthetic  $\beta$ -lactam antibiotics that are competitive with traditional chemical condensation approaches.

Here, we describe the synthetic properties of a new penicillin acylase, PAS2, which was obtained by functional screening of an environmental gene bank constructed from a sand soil enrichment culture. Initial experiments revealed a higher preference towards the synthesis reaction for semi-synthetic penicillins as compared to the enzyme isolated from *Escherichia coli* ATCC 11105, which is the best studied penicillin G acylase to date and which we use as a benchmark [13,14]. In this paper, we present a comprehensive kinetic study of this interesting new enzyme and show that its use in the production of semi-synthetic penicillins could lead to more efficient processes than with *E. coli* PA.

## 2. Material and methods

### 2.1. Cloning of *pas2* from a sandy soil enrichment culture

The construction of environmental gene banks and the screening procedure for amidase-producing transformants will be described in detail elsewhere. Briefly, organisms present in a sandy soil sample were grown under selective

pressure, supplying D-phenylglycine amide (PGA) as a sole source of nitrogen. Genomic DNA was isolated from this enrichment culture and used to construct an environmental gene bank in the leucine auxotrophic host strain *E. coli* TOP10 (Invitrogen), using plasmid pZero-2 (Invitrogen) as a vector. Selection of clones able to utilize D-phenylacetyl-L-leucine as a source of leucine [15] was carried out on selective agar plates and yielded two different recombinants. One of the clones also exhibited activity towards 2-nitro-5-[(phenylacetyl)amino]-benzoic acid (NIPAB), a well-known colorimetric substrate of PAs [16]. Sequencing and further substrate profiling revealed that S2 indeed encoded a PA, which was named PAS2. The DNA sequence of the *pas2* gene was submitted to GenBank as accession number AY573298.

### 2.2. Subcloning of the *pas2* gene

To achieve high-level expression of PAS2, *pas2* was cloned behind the tightly regulatable P<sub>BAD</sub> promoter located on pBAD/Myc-HisA-*Nde*I. This plasmid vector solely differs from the commercially available vector pBAD/Myc-HisA (Invitrogen) by carrying an *Nde*I instead of an *Nco*I recognition sequence in its multiple cloning site. The *pas2* gene was cloned including its own signal sequence, using a forward PCR primer based on the 5'-end of the gene with an introduced *Nde*I restriction site (underlined, start codon bold), 5' ttggagacagagcatatgaagcagcattgttg 3', and a reverse primer based on the 3'-end of the gene with a *Sal*I site (underlined) incorporated (5' ccaggcgctgcacacggtcagtagcg 3'). PCR amplification was carried out with pWO polymerase (Roche) under standard conditions, using whole cells of the original clone S2 as a template. PCR product and vector were digested with *Nde*I/*Sal*I and *Nde*I/*Xho*I, respectively, and ligated with T4 ligase according to the instructions of the manufacturer (Roche). The ligation mixture was transformed to electro-competent *E. coli* TOP10 cells, and the construct (pBAD-PAS2) was confirmed by sequencing.

### 2.3. Protein purification

*E. coli* PA was purified as described before [13]. The obtained enzyme solution was concentrated by ultrafiltration (Amicon bioseparations, YM 30 filter) before the enzyme was rebuffed in 50 mM potassium phosphate buffer (pH 7.0) with 5% glycerol, using an Econo-pac 10DG column (BioRad).

For PAS2, a similar purification scheme was used. *E. coli* TOP10 (pBADPAS2) cells were grown in LB [17] at 17 °C with rotary shaking at 200 rpm. To induce protein expression from P<sub>BAD</sub>, the medium was supplied with 0.8% arabinose after 2 days of growth. After another 24 h of incubation, cells were harvested by centrifugation at 5000  $\times$  g for 10 min (4 °C). To prepare a periplasmatic extract, cells were resuspended in 1/10 of the original culture volume of ice-cold osmotic shock buffer (20% sucrose, 100 mM Tris-HCl, 10 mM EDTA; pH 8.0) and centrifuged as described above. Cell walls

were disrupted by resuspending the cell pellet in 1/10 of the original culture volume of ice-cold 1 mM EDTA. After centrifugation ( $6000 \times g$ , 15 min,  $4^\circ\text{C}$ ), 1 M potassium phosphate buffer (pH 7.0) was added to the supernatant (periplasmic extract) to a final concentration of 50 mM. Subsequently, solid  $(\text{NH}_4)_2\text{SO}_4$  was added to a final concentration of 1.5 M, while stirring at  $4^\circ\text{C}$ . The solution was subjected to hydrophobic interaction chromatography, using a Resource Phe column (Amersham Pharmacia Biotech), and eluted with a linear gradient of 1.5–0 M  $(\text{NH}_4)_2\text{SO}_4$  in 20 mM potassium phosphate buffer (pH 7.0). PAS2 eluted at a concentration of 300 mM  $(\text{NH}_4)_2\text{SO}_4$ . Fractions containing enzyme activity were pooled, concentrated and rebuffed as described for *E. coli* PA. About 10 mg PAS2 could be obtained per liter of culture grown as explained above. The purity of the enzyme was  $>95\%$  as judged by SDS-PAGE. The enzyme was stored at  $-20^\circ\text{C}$  and could be defrosted several times without detectable loss of activity.

The amount of active enzyme in penicillin acylase preparations was determined by titration with the irreversible inhibitor phenylmethylsulfonyl fluoride (Boehringer Mannheim) as done by Alkema et al. [18].

#### 2.4. Mass spectrometry

The molecular masses of the two subunits of PAS2 were determined at the Mass Spectrometry Core Facility, University of Groningen (The Netherlands), using an electrospray triple quadrupole mass spectrometer (API 3000, PE-Sciex). Full-scan spectra were recorded with a step size of 0.1 amu and analyzed with Biomultiview software (version 1.5, PE-Sciex). For this experiment, the buffer system of the original enzyme solution was replaced by a 10 mM ammonium acetate buffer (pH 6.8) with an Econo-pac 10DG column (BioRad) and 0.1% formic acid was added before analysis.

#### 2.5. Kinetic measurements

All enzymatic conversions were carried out in 50 mM potassium phosphate buffer (pH 7.0) at  $30^\circ\text{C}$ . Steady-state kinetic parameters for the hydrolysis of the colorimetric substrates 2-nitro-5-[(phenylacetyl)amino]-benzoic acid and D-2-nitro-5-[(phenylglycyl)amino]-benzoic acid (NIPGB) were determined by measuring initial velocities of 5-amino-2-nitro-benzoic acid release ( $\Delta\epsilon_{405\text{ nm}} = 9.09\text{ mM}^{-1}\text{ cm}^{-1}$ ) at 405 nm in a Perkin-Elmer Bio40 UV-vis spectrometer, using substrate concentrations ranging from 5  $\mu\text{M}$  to 10 mM. Data were fitted with the program Origin 6.0 (Microcal Software, Inc.).  $K_i$  values for phenylacetic acid and  $K_m$  values for non-colorimetric substrates were determined by measuring the inhibition on the hydrolysis of NIPGB as described by Alkema et al. [19]. The  $k_{\text{cat}}$  values were determined separately by monitoring the initial velocities of substrate conversion at substrate concentrations of at least  $10 \times K_m$  by high-performance liquid chromatography (HPLC). Product concentrations were determined at several times in order to obtain at least three data points in the initial phase of con-

version. All HPLC analyses were carried out using a 10-cm Chrompack C18 column (5 mm diameter) in connection with Jasco PU-980 pumps and a Jasco MD-910 detector set at 214 nm. Compounds were isocratically eluted at a flow rate of  $1\text{ ml min}^{-1}$  with a solution of  $340\text{ mg l}^{-1}$  sodium dodecylsulfate and  $680\text{ mg l}^{-1}$   $\text{KH}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$  in a 30:70 (v/v) acetonitrile/water mixture of pH 3.0 (adjusted with diluted phosphoric acid).

Kinetically controlled enzymatic synthesis of  $\beta$ -lactam antibiotics at pH 7.0 was carried out by mixing enzyme with solutions of activated acyl donor [either phenylacetamide (PAA), D-phenylglycine amide, *p*-hydroxyphenylacetamide (HPAA) or D-*p*-hydroxyphenylglycine amide (HPGA)] and an appropriate  $\beta$ -lactam compound (6-APA or 7-ADCA). The initial concentration of acyl donor was 15 mM in all experiments, whereas the concentration of  $\beta$ -lactam acyl acceptor varied between 1 and 190 mM. All reactants were monitored in time by HPLC analysis and initial rates of formation of the antibiotic ( $v_{\text{ps}}$ ) and the hydrolyzed acyl donor ( $v_{\text{ph}}$ ) were determined. Peak areas were related to the concentration of the respective compounds by calibration curves that were established with solutions of the pure compounds. Only for *p*-hydroxyphenicillin G, no authentic response factor could be determined due to the lack of a commercially available reference compound. However, as response factors ( $\text{mM}^{-1}$ ) of compounds varying only by the presence of a *p*-hydroxy group (PGA and HPGA, ampicillin and amoxicillin, and cephalixin and cefadroxil), were found to be very similar ( $<10\%$  difference), we approximated the response given by *p*-hydroxyphenicillin G with the one obtained for penicillin G.

#### 2.6. Chemicals

Ampicillin, cefadroxil, and cephalixin were purchased from Sigma; HPAA was from Acros Organics. Penicillin G, amoxicillin, 7-ADCA, 6-APA, PGA, and HPGA were provided by DSM Life Sciences (Delft, The Netherlands). NIPAB and NIPGB were synthesized by reacting phenylacetic acid chloride and D-phenylglycine chloride, respectively, with 5-amino-2-nitro-benzoic acid in a water/acetone mixture. PAA was prepared by adding phenylacetylchloride drop wise to a concentrated ammonia solution, resulting in the formation of a white precipitate, which was filtered off and dried to constant weight. D-Phenylacetyl-L-leucine was obtained through standard organic chemical peptide coupling chemistry. During the synthesis, consecutive washing of the di-protected dipeptide with acid and base ensured that absolutely no free leucine remained in the sample.

### 3. Results and discussion

#### 3.1. Isolation of the new penicillin acylase PAS2

From an enrichment culture for amidase-containing organisms inoculated with sand soil and supplied with

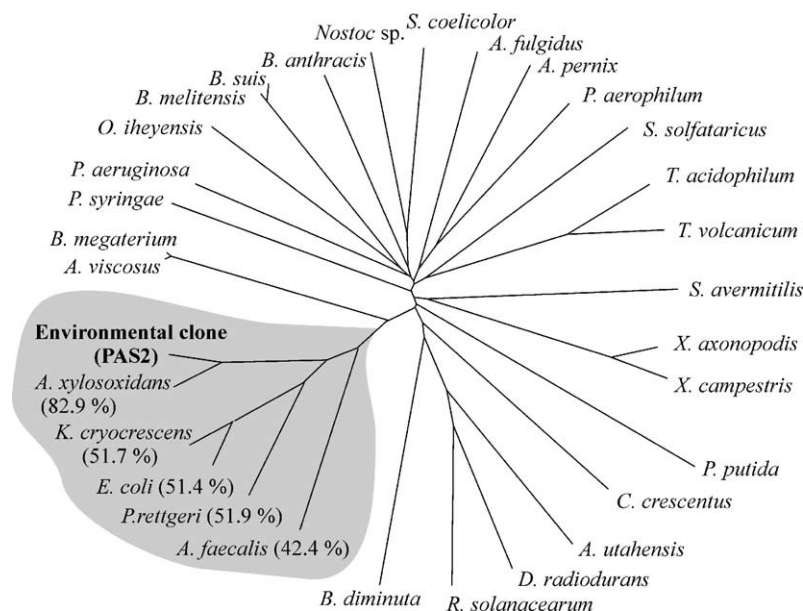


Fig. 2. Phylogeny of penicillin acylases. The new enzyme PAS2 falls into a group of closely related enzymes with experimentally verified PA activity (>40% homology, shaded in gray). Numbers in brackets indicate amino acid sequence identity with PAS2. Sequences identified by BLAST searches [20] with PAS2 as the query were aligned with Megalign software (DNASTar Inc.) and represented in a tree structure with Treeview software [24].

D-phenylglycine amide as a sole source of nitrogen, genomic DNA was extracted and cloned into a high-copy plasmid vector. Recombinant plasmids were transformed to the leucine-auxotroph *E. coli* host strain TOP10. The clone expressing PAS2 was identified by growth on a selective medium, to which phenylacetyl-L-leucine was added as the only leucine supply.

The coding sequence of PAS2 was at first identified by sequence analysis of the 7.2 kb insert carried by the original clone and subsequently confirmed by subcloning in the expression vector pBAD/Myc-HisA\_NdeI, which allowed high-level expression of the active protein. A BLAST search [20] with the derived protein sequence yielded a proposed penicillin G acylase of *Achromobacter xylosoxidans* as the strongest hit (82.9% identity, accession AAP20806) and revealed somewhat lower sequence similarity to a group of closely related and well-studied enzymes (Fig. 2), including the PA of *E. coli* ATCC 11105 (51.4% identity, accession AAA24324). Sequence analysis and comparison with other PAs suggested the expression of PAS2 as a preproprotein, composed of a signal peptide that leads to the translocation of the protein to the periplasm while being cleaved off itself, and two subunits that are separated by a spacer peptide. In *E. coli*, this spacer is removed in the periplasm initiated by an intramolecular autoproteolytic process [21]. Mass spectrometric analysis of the purified mature protein confirmed the cleavage of the PAS2 preproprotein between positions 24 and 25, which was also the cleavage site predicted by the PSORT program [22] due to the presence of positively charged residues at the N-terminus followed by hydrophobic residues and a consensus pattern for recognition by signal peptidase I (AXA) at the C-terminus of the signal peptide (MKQHLLSAAILAACAGVGAAPAH-

QS...). About 30% of the  $\alpha$ -subunit, however, was found to have a 128 Da lower mass. This mass difference corresponds to the molecular weight of a glutamine residue, suggesting that cleavage by the *E. coli* signal peptidase is not very specific and can also occur between positions 25 and 26.

Due to the obtained molecular masses of the small subunit, the exact cleavage point between the  $\alpha$ -subunit C-terminus and the spacer could be determined. The removal of the 54 amino acid spacer was found to result in the release of a 229 amino acid  $\alpha$ -subunit (25.5 kDa) and a  $\beta$ -subunit of 555 amino acids (61.9 kDa). Hydrolytic activity on the colorimetric penicillin acylase substrate NIPAB was mainly found in periplasmic extracts and not in the cytosolic or membrane fraction of cells, which supports the idea that PAS2 is processed in a similar way as the *E. coli* PA. The proposed cleavage also yields an N-terminal serine on the  $\beta$ -subunit, which is responsible for the catalytic activity of the enzyme as confirmed by the stoichiometric inactivation of PAS2 by phenylmethylsulfonyl fluoride. Because of the presence of this N-terminal serine that can act as a nucleophile, the observed gene homology and topology, and the activation of the protein by a presumably autocatalytic process, we conclude that PAS2 is a new member of the Ntn-hydrolase superfamily [23]. The characteristic  $\alpha\beta\alpha$ -fold of this class of enzymes was predicted to be present in PAS2 as well when a homology model was made using the structure of *E. coli* PA as a template.

### 3.2. Biocatalytic performance

The enzymatic hydrolysis of various activated acyl donors, antibiotics, and colorimetric substrates that are typically con-



Table 1  
Steady-state kinetic parameters of PAS2 and *E. coli* PA<sup>a</sup> for different substrates<sup>b</sup>

Compound	PAS2			<i>E. coli</i> PA		
	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_{\text{m}}$ (mM)	$k_{\text{cat}}/K_{\text{m}}$ (mM <sup>-1</sup> s <sup>-1</sup> )	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_{\text{m}}$ (mM)	$k_{\text{cat}}/K_{\text{m}}$ (mM <sup>-1</sup> s <sup>-1</sup> )
NIPAB	24	0.004	6000	18	0.015	1200
NIPGB	12	0.646	18.6	14	1.3	10.8
PAA	23	0.030	767	46	0.156	295
HPAA	29	0.027	1074	47	0.114	412
PGA	25	12.0	2.1	57	30	1.9
HPGA	16	9.1	1.8	28	12.2	2.3
Penicillin G	25	0.012	2083	39	0.013	3000
Ampicillin	16	0.575	27.8	25	2.5	10.0
Amoxicillin	15	0.399	37.6	17	1.07	15.9
Cephalexin	20	1.3	15.4	29	1.5	19.3
Cefadroxil	13	0.284	45.8	32	0.642	49.8

<sup>a</sup> Coefficients of variation were below 10% for all data.

<sup>b</sup> NIPAB, 2-nitro-5-[(phenylacetyl)amino]-benzoic acid; NIPGB, D-2-nitro-5-[(phenylglycyl)amino]-benzoic acid; PAA, phenylacetamide; HPAA, *p*-hydroxyphenylacetamide; PGA, D-phenylglycine amide; and HPGA, *D-p*-hydroxyphenylglycine amide.

verted by PAs was studied (Table 1). Due to its primary activity against penicillin G, PAS2 can be classified as a penicillin G acylase (type II PA [4]) although it can convert a much broader range of  $\beta$ -lactam antibiotics. In general, substrate specificities ( $k_{\text{cat}}/K_{\text{m}}$ ) were found to be similar or higher than for the *E. coli* PA, with comparatively stronger preference of substrates lacking an  $\alpha$ -amino substituent.

Compared to the *E. coli* enzyme, PAS2 was found to be much more susceptible towards competitive inhibition by phenylacetic acid ( $K_{\text{i}} = 14 \mu\text{M}$  versus  $50 \mu\text{M}$  for *E. coli* PA). Taking into account also its lower specificity for penicillin G, the new enzyme appears to be of limited use for the hydrolytic production of 6-APA from penicillin G where phenylacetic acid is stoichiometrically released. In the hydrolysis of NIPAB, however, PAS2 was found to be about 5-fold more effective, which makes the colorimetric compound PAS2's best substrate tested so far.

### 3.3. Kinetically controlled antibiotic synthesis

In contrast to hydrolysis, the performance of PAS2 in the synthesis of penicillin G from 15 mM PAA and 25 mM 6-

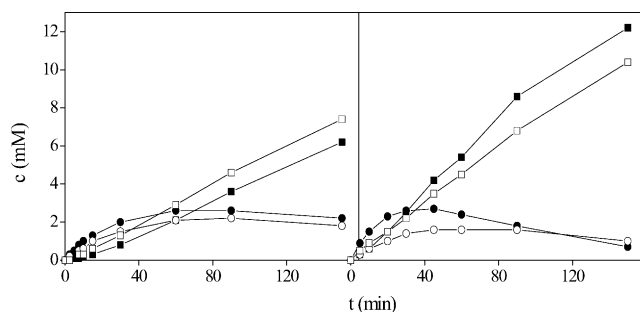


Fig. 3. Kinetically controlled synthesis of ampicillin (left) and amoxicillin (right) using 25 mM 6-aminopenicillanic acid (6-APA) and 15 mM D-phenylglycine amide (PGA) or D-*p*-hydroxyphenylglycine amide (HPGA), respectively. PAS2: (●) antibiotic, (■) D-phenylglycine (PG, left) and D-*p*-hydroxyphenylglycine (HPG, right). *E. coli* PA: (○) antibiotic, (□) PG (left) and HPG (right).

APA at pH 7.0 was found to be significantly better than that of the *E. coli* enzyme, allowing a more than 2-fold higher maximal penicillin G accumulation in the course of reaction (data not shown). Under the same reaction conditions, also 1.2-fold more ampicillin and 1.6-fold more amoxicillin could be produced with PAS2 (Fig. 3). With PAS2, reactions proceeded at 51% (ampicillin) and 106% (amoxicillin) higher pace, measured as the initial rate of antibiotic formation. Because of these promising first results, the new enzyme was studied in more detail, in particular with respect to the production of the semi-synthetic antibiotics ampicillin, amoxicillin, cephalexin, and cefadroxil.

Kinetically controlled antibiotic synthesis with PA follows the kinetic scheme represented in Fig. 4. Besides the desired condensation of the activated acyl donor with the nucleophilic  $\beta$ -lactam compound (N) to the semi-synthetic antibiotic (Ps), PA also catalyzes the nucleophilic attack of water on AD and Ps, leading to the formation of the acyl donor acid as a hydrolytic side-product (Ph). In the beginning of the reaction, however, product hydrolysis can be neglected and the

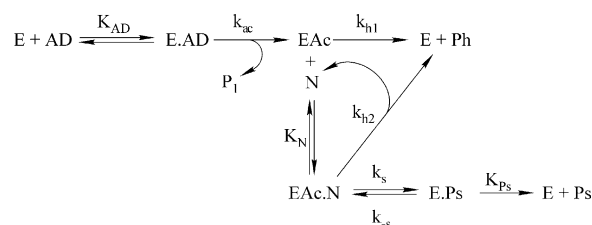


Fig. 4. Kinetic scheme of the synthesis and hydrolysis reactions catalyzed by penicillin acylase via the formation of a covalent acyl-enzyme intermediate EAc. Designations: E (free enzyme), AD (activated acyl donor), EAD (non-covalent enzyme-acyl donor complex), P<sub>1</sub> (first reaction product that is released upon acylation of the enzyme), Ph (product of hydrolysis reactions), N ( $\beta$ -lactam nucleophile), EAc.N (covalent acyl-enzyme intermediate with non-covalently bound nucleophile), Ps (product of the synthesis reaction, i.e. the antibiotic), E.Ps (enzyme-antibiotic complex). Rate constants  $k_{\text{ac}}$ ,  $k_{\text{h1}}$ ,  $k_{\text{h2}}$ ,  $k_{\text{s}}$ , and  $k_{-\text{s}}$  refer to different reaction steps as indicated in the scheme.  $K_{\text{AD}}$ ,  $K_{\text{Ps}}$  and  $K_{\text{N}}$  are the binding constants of AD and Ps to the free enzyme and of N to the acyl-enzyme intermediate, respectively.

formation of Ph is solely due to hydrolysis of the activated side chain donor. The ratio of the initial rates of antibiotic synthesis ( $v_{Ps}$ ) and AD hydrolysis ( $v_{Ph}$ ), the so-called synthesis/hydrolysis ratio, therefore reflects the tendency of the covalent acyl-enzyme intermediate to react with the  $\beta$ -lactam compound instead of with water.

The initial synthesis/hydrolysis ratio is given by:

$$\left(\frac{v_{Ps}}{v_{Ph}}\right)_{\text{ini}} = \frac{1}{\gamma} \frac{[N]}{1/\beta_0\gamma + [N]} \quad (1)$$

with the complex kinetic parameters  $\beta_0$  and  $\gamma$ . From the kinetic scheme, it follows that  $\beta_0 = k_s/(k_{h1}K_N)$  and  $\gamma = k_{h2}/k_s$  [12]. As can be seen from Eq. (1), the tendency towards the synthesis reaction rises with increasing concentration of the  $\beta$ -lactam compound in a hyperbolic, Michaelis–Menten-type way. Consequently,  $1/\gamma$  corresponds to the maximal synthesis/hydrolysis ratio that can be reached under certain reaction conditions and describes the mode of conversion when the acyl-enzyme intermediate is fully complexed with the nucleophilic  $\beta$ -lactam compound (EAc.N, Fig. 4). At low nucleophile concentrations, when most of the acyl-enzyme intermediate is still free (EAc), the synthesis/hydrolysis ratio linearly increases with the nucleophile concentration according to:

$$\left(\frac{v_{Ps}}{v_{Ph}}\right)_{\text{ini}} = \beta_0[N] \quad (2)$$

We determined initial synthesis/hydrolysis rate ratios in the synthesis of a number of antibiotics, using a wide range of different  $\beta$ -lactam nucleus concentrations (Fig. 5). In the resulting  $(v_{Ps}/v_{Ph})_{\text{ini}}$  versus  $[N]$  plots,  $\beta_0$  constitutes the initial slope, whereas  $1/\gamma$  is the asymptotic value to which the curve saturates at high  $\beta$ -lactam nucleophile concentrations. PAS2 reached clearly higher synthesis/hydrolysis ratios over the whole concentration range for all 6-APA derived antibiotics tested. In the synthesis of penicillin G by *E. coli* PA and cefadroxil by either of the two studied enzymes, no saturation was observed even at the highest feasible  $\beta$ -lactam concentrations, compromising the determination of  $\gamma$ . In contrast,  $\beta_0$  could be readily determined for all antibiotics and turned out to be significantly higher for 6-APA in PAS2- than in *E. coli* PA-catalyzed reactions (Fig. 5, A and B). Remarkably, 7-ADCA reactivity was almost identical for both studied enzymes irrespective of the kind of acyl donor used (Fig. 5C).

To fully describe the catalytic behavior of penicillin acylase, a third parameter,  $\alpha$ , is required [12]. This parameter delimits the maximal amount of antibiotic that can be accumulated in the course of reaction and describes the tendency of the enzyme to hydrolyze the initially formed product. Basically,  $\alpha$ , quantifies the enzyme's preference to hydrolysis of the antibiotic over hydrolysis of the activated acyl donor:

$$\alpha = \frac{(k_{\text{cat}}/K_m)_{\text{Ps}}}{(k_{\text{cat}}/K_m)_{\text{AD}}} \quad (3)$$

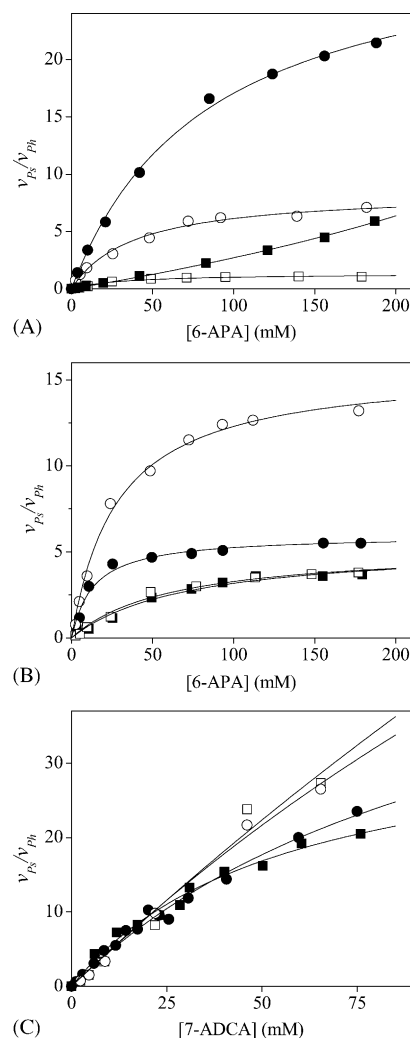


Fig. 5. Nucleophile reactivity of 6-aminopenicillanic acid (6-APA) and 7-aminodesacetoxycephalosporanic acid (7-ADCA) in the synthesis of different antibiotics. Acyl donors were used at an initial concentration of 15 mM. (A) Synthesis of penicillin G by PAS2 (●) and *E. coli* PA (■) and production of *p*-hydroxyphenylpenicillin G by PAS2 (○) and *E. coli* PA (□). (B) Synthesis of ampicillin by PAS2 (●) and *E. coli* PA (■) and production of amoxicillin by PAS2 (○) and *E. coli* PA (□). (C) Synthesis of cephalexin by PAS2 (●) and *E. coli* PA (■) and production of cefadroxil by PAS2 (○) and *E. coli* PA (□).

To allow high product accumulation, the specificity for the acyl donor should be as high as possible with a low reactivity towards the desired synthesis product, i.e.  $\alpha$  should be small.

Table 2 summarizes the complex kinetic parameters determined for PAS2 and *E. coli* PA. As observed for the nucleophile reactivity, differences in  $\alpha$  between the two studied enzymes were more clearly expressed for 6-APA than for 7-ADCA-derived antibiotics, which suggests that the binding mode of 6-APA in the active site is altered in PAS2 while being similar to that in *E. coli* PA for 7-ADCA. However, amino acid residues that have previously been identified to be important in  $\beta$ -lactam binding in the *E. coli* enzyme [13,19] are conserved in PAS2. Consequently, subtle differences in the orientation of these residues or additional and not yet iden-

Table 2

Complex kinetic parameters  $\alpha$ ,  $\beta_0$ , and  $\gamma$ , characterizing the efficiencies of PAS2 and *E. coli* PA in antibiotic synthesis

Acyl donor (AD)	$\beta$ -Lactam nucleophile (N)	Antibiotic (Ps)	$\alpha$		$\beta_0$ ( $M^{-1}$ )		$1/\gamma^a$		$P_{smax}$ (mM) <sup>b</sup>	
			PAS2	<i>E. coli</i> PA	PAS2	<i>E. coli</i> PA	PAS2	<i>E. coli</i> PA	PAS2	<i>E. coli</i> PA
PAA	6-APA	Penicillin G	2.7	10.2	373	30	31	N.D.	1.8	0.8
HPAA	6-APA	<i>p</i> -Hydroxyphenicillin G	N.D.	N.D.	204	35	9	1.5	N.D.	N.D.
PGA	6-APA	Ampicillin	13.2	5.3	448	76	6	5	2.4	2.0
HPGA	6-APA	Amoxicillin	20.9	6.9	560	86	16	5	2.7	1.7
PGA	7-ADCA	Cephalexin	7.3	10.2	509	638	59	36	4.1	3.7
HPGA	7-ADCA	Cefadroxil	25.4	21.7	444	427	N.D.	N.D.	2.4	2.2

N.D., not determined. PAA, phenylacetamide; HPAA, *p*-hydroxyphenylacetamide; PGA, D-phenylglycine amide; HPGA, D-*p*-hydroxyphenylglycine amide; 6-APA, 6-aminopenicillanic acid; and 7-ADCA, 7-aminodesacetoxycephalosporanic acid.

<sup>a</sup>  $1/\gamma$  corresponds to the maximal initial synthesis/hydrolysis ratio that can be reached in the synthesis of a given antibiotic.

<sup>b</sup> Maximal level of accumulation of the synthesis product obtained with 25 mM of  $\beta$ -lactam nucleophile and 15 mM of acyl donor.

tified residues are expected to be responsible for observed deviations in binding of the  $\beta$ -lactam nucleophile. Besides altered binding of the incoming nucleophilic  $\beta$ -lactam group, binding of the acyl donor is also different in PAS2, which is most obvious for phenylacetamide. For this compound, a 5-fold higher apparent affinity was observed in PAS2, leading to a strongly improved  $\alpha$  parameter for the new enzyme. Together with the high reactivity of 6-APA, this fact allowed the accumulation of 2.3 times more penicillin G than with *E. coli* PA.

Experimental data of product concentrations ([Ps] and [Ph]) in the course of the reaction were in good agreement with the theoretically expected ones calculated on the basis of the kinetic model (Eq. (4)) as is exemplified for ampicillin and amoxicillin in Fig. 6. These results show that the model, which was originally developed for *E. coli* PA, also applies to PAS2.

$$\frac{d[Ps]}{d[Ph]} = \frac{\beta_0[N][AD] - \alpha[Ps](1 + \beta_0\gamma[N])}{(1 + \beta_0\gamma[N])([AD] + \alpha[Ps])}, \quad \text{with } [AD]_0 = [AD] + [Ps] + [Ph] \text{ and } [N]_0 = [N] + [Ps](4)$$

By using this model, product yields can also be calculated for higher substrate concentrations, which may be more relevant for industrial processes. As can be seen from Fig. 7, the relative performance of PAS2 in ampicillin as well as amoxicillin synthesis is most clearly improved compared to the *E. coli* PA in the low 6-APA region (<100 mM) due to the significantly

higher  $\beta_0$  parameters of PAS2. When higher concentrations of both reactants are used, however, relative differences in maximal antibiotic yield decrease, and for ampicillin the accumulation level is even somewhat lower than with the *E. coli* enzyme. For the production of amoxicillin, in contrast, PAS2 is more effective over the whole range of substrate concentrations. Relative improvements range from 240% in the low PGA/low 6-APA region to about 14% at high substrate concentrations. This increase in synthetic performance would allow the use of lower concentrations of substrates, particularly 6-APA, in the production process. To reach a 48.5 mM amoxicillin concentration, for instance, which is the maximum achievable with *E. coli* PA under the modeled conditions, only 100 mM instead of 200 mM 6-APA would be required with PAS2 at a HPGA concentration of 200 mM (Fig. 7).

In conclusion, high specific activity combined with improved kinetic properties that allow increased levels of antibiotic accumulation constitute the main advantages of the newly isolated enzyme in the kinetically controlled production of semi-synthetic penicillins, particularly of amoxicillin. The observed high turnover rates are especially remarkable

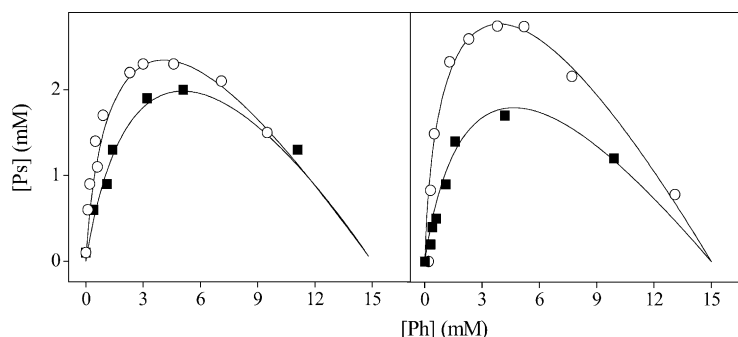


Fig. 6. Product formation in the course of ampicillin (left) and amoxicillin (right) synthesis, using PAS2 (○) and *E. coli* PA (■). Ps is the synthetic product, i.e. the antibiotic, and Ph is the hydrolyzed acyl donor, i.e. D-phenylglycine (PG, left) or D-*p*-hydroxyphenylglycine (HPG, right). All reactions were carried out with 15 mM acyl donor and 25 mM  $\beta$ -lactam nucleophile. Solid lines were calculated by numerical integration of Eq. (4), using the experimentally determined parameters and substrate concentrations given in Table 2.



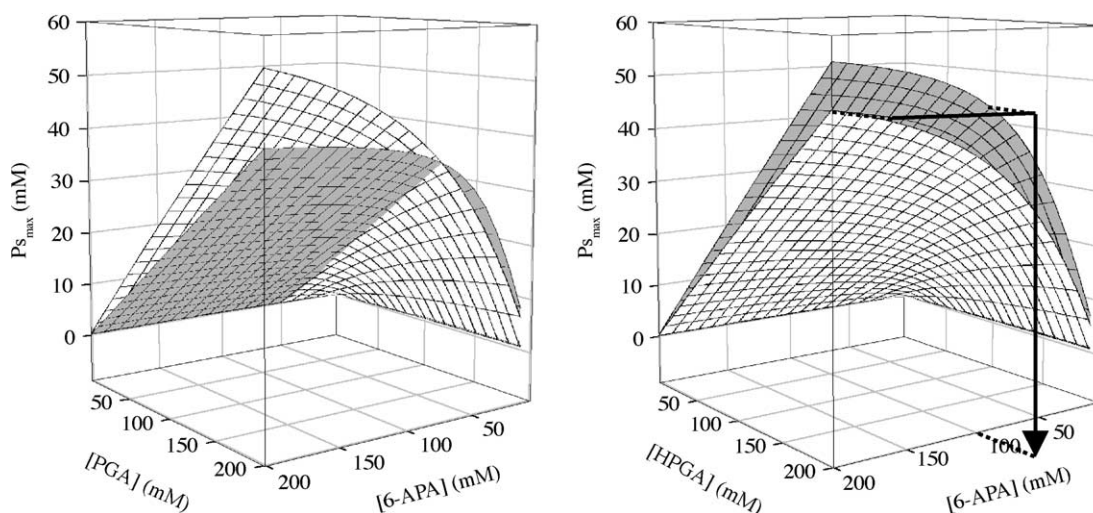


Fig. 7. Maximal level of accumulation  $P_{s_{\max}}$  of ampicillin (left) and amoxicillin (right) predicted for different substrate concentrations with PAS2 (gray) and *E. coli* PA (white). The arrow indicates the concentration of 6-aminopenicillanic acid (6-APA) that allows the accumulation of the same amount of amoxicillin as maximally obtained with *E. coli* PA (at 200 mM HPGA). PGA, D-phenylglycine amide; HPGA, D-p-hydroxyphenylglycine amide.

since improved synthetic capacity has been found to be coupled to a loss of enzyme activity for site-directed mutants of *E. coli* PA [13,19]. The possibilities of further improvement of PAS2 by mutagenesis techniques are currently under investigation. The discovery of PAS2 demonstrates once more that the steadily rising request for improved biocatalysts may in part be satisfied by screening the almost untapped environmental gene pool.

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